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CHAPTER

2

Why Do We Produce Anti-Gal: Evolutionary Appearance of Anti-Gal in Old World Primates

OUTLINE

INTRODUCTION

Anti-Gal is produced in humans throughout life in large amounts, as ~1% of immunoglobulins (Galili et al., 1984), and it binds specifically a mammalian carbohydrate antigen called the α-gal epitope with the structure Galα1-3Galβ1-4GlcNAc-R (Galili et al., 1985). Moreover, ~1% of B lymphocytes in human blood are capable of producing this antibody (Galili et al., 1993). The dedication of such a significant proportion of B cell clones to the production of one antibody raises the question whether anti-Gal has a distinct physiologic role in humans. At present, there is no clear answer to this question. The removal of senescent red blood cells (RBC) seems to be

The Natural Anti-Gal Antibody as Foe Turned Friend in Medicine
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associated with binding of anti-Gal to a cryptic antigen that is exposed on circulating RBC when they reach the age of ~120 days (Galili et al., 1986a) and in some pathologic RBC, at an earlier age of the cell (Galili et al., 1983, 1986b). However, this mechanism is not applicable to New World monkeys, lemurs, and all other nonprimate mammals. The geographic distribution of anti-Gal only in Old World monkeys, apes, and humans (collectively called *catarrhines* [nostrils pointing downwards]) raises the possibility that this antibody has protected against pathogens present only in the landmass of Eurasia and Africa (the "Old World") (Galili et al., 1987a,b, 1988a). New World monkeys (called *platyrrhines* ["flat" nose with nostrils pointing sideward]) and lemurs, all synthesize α-gal epitopes and lack the anti-Gal antibody (Galili et al., 1987a, 1988a). These primates have not been reported to display higher susceptibility to infections, in comparison with Old World monkeys and apes, when kept in zoos in Asia, Africa, or Europe. This suggests that anti-Gal is not required for current protection against any particular pathogen endemic to the Old World. It has been suggested that anti-Gal may protect humans against enveloped viruses originating in nonprimate mammals and presenting α-gal epitopes, by binding to these epitopes and inducing neutralization and destruction of the viruses presenting them (Repik et al., 1994; Rother et al., 1995; Takeuchi et al., 1996, 1997). However, it has not been proven as yet that anti-Gal has a current vital protective role. Nevertheless, one may assume that the striking distribution of anti-Gal only in Old World monkeys, apes, and humans versus the synthesis of α-gal epitopes in all other mammals are associated with a major selective event in the course of Old World primate (i.e., *catarrhines*) evolution.

As detailed in Chapter 1, the glycosylation enzyme synthesizing α-gal epitopes in mammals is α1,3galactosyltransferase (α1,3GT) that was originally found in cells of rabbit (Basu and Basu, 1973; Betterige and Watkins, 1983) and subsequently in mouse, cow, and New World monkey cells (Blake and Goldstein, 1981; Blanken and van den Eijnden, 1985; Galili et al., 1988a). Studies on the expression of the biosynthetic product of α1,3GT, i.e., the α-gal epitope, in various mammals further imply that α1,3GT is active in nonprimate mammalian cells, lemurs, and New World monkeys, and it is absent in Old World monkeys, apes, and humans (Galili et al., 1987a, 1988a; Oriol et al., 1999). Furthermore, synthesis of the α-gal epitope in both marsupial and placental mammals and its absence in other vertebrates implies that the α1,3GT gene (also called *GGTA1*) and the α-gal epitope synthesized by the enzyme encoded by this gene, appeared early in mammalian evolution before marsupial and placental lineages separated from a common ancestor (Fig. 1). Since its appearance >100 million years ago, the α-gal epitope has been continuously synthesized, and it is being synthesized in nonprimate mammals. Synthesis of α-gal epitopes by α1,3GT has been conserved also in lemurs that evolved in the island of Madagascar and in New World monkeys that evolved in the South American continent, both isolated from the Old World by oceanic barriers. In contrast, α-gal epitopes are not synthesized in Old World monkeys, apes, and humans, all of which lack α1,3GT activity (Fig. 1) and all have evolved in the Old World continents of Asia, Africa, and Europe (Galili et al., 1987a, 1988a). As further discussed below, these Old World primates have the α1,3GT gene as a mutated pseudogene (Larsen et al., 1990; Galili and Swanson, 1991; Koike et al., 2002; Lantéri et al., 2002). These observations suggest that ancestral Old World primates synthesized in the distant past the α-gal epitope, similar to nonprimate mammals, lemurs, and New World monkeys. However, at a certain evolutionary period, after the geographic separation between Old World primates and New World monkeys (estimated to occur ~30 million years ago [mya]) (Dawkins, 2004; Steiper and Young, 2006; Schrago, 2007),

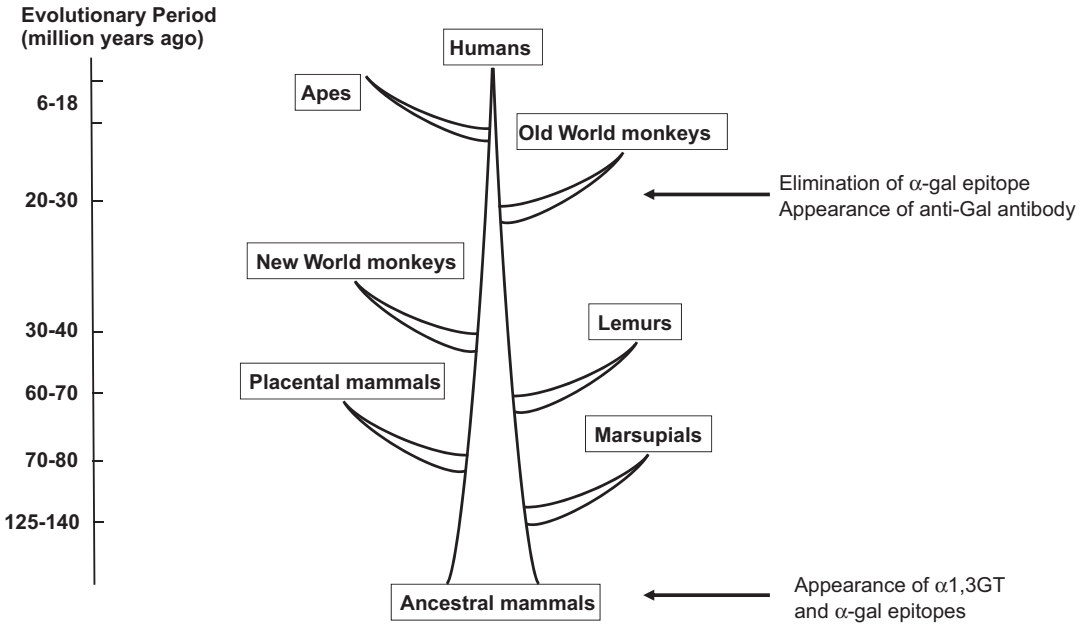

FIGURE 1 A schematic evolutionary tree describing the estimated evolutionary period in which α1,3galactosyltransferase and the α-gal epitope appeared in early mammals, and the period in which the selective pressure for elimination of primates synthesizing α-gal epitopes initiated (indicated by *arrows*). The estimated evolutionary periods for divergence events in mammals are indicated on the left. The absence of the α-gal epitope in vertebrates that are not mammals, and its synthesis in nonprimate mammals implies that α1,3GT and the α-gal epitope appeared in mammals prior to the split between marsupial and placental mammals. The absence of α1,3GT and α-gal epitopes only in Old World monkeys, apes, and humans implies that inactivation of the α1,3GT gene (*GGTA1*) and elimination of α-gal epitopes occurred after the split between New World monkeys and Old World primates. The estimates of the evolutionary periods of divergence in mammals are based on several studies (Dawkins, 2004; Schrago, 2007; Steiper and Young, 2006). *Adapted from Galili, U., 2016. Natural anti-carbohydrate antibodies contributing to evolutionary survival of primates in viral epidemics? Glycobiology 26, 1140–1150, with permission.*

an evolutionary selective process in primate populations resulted in extinction of Old World primates that synthesized α-gal epitopes. This extinction was followed by the expansion of monkey and ape populations with inactivated α1,3GT gene, which lacked α-gal epitopes, and thus could produce the natural anti-Gal antibody. This transition from α-gal epitope synthesis to elimination of primates synthesizing this carbohydrate antigen and the appearance of primates producing an antibody against the α-gal epitope is observed only in Old World primates. Such observation raises the possibility that this evolutionary event was associated with a selection process mediated by a detrimental pathogen that was endemic to the Old World. Although it is practically impossible to indentify pathogens that affected evolution of primates millions of years ago, this chapter describes several scenarios that are most likely to explain these evolutionary events in ancestral Old World primates. Understanding anti-Gal evolution requires a short discussion on production of the group of antibodies called "natural anti-carbohydrate antibodies" in response to antigenic stimulation by gastrointestinal (GI) bacteria.

NATURAL ANTI-CARBOHYDRATE ANTIBODIES TO BACTERIAL ANTIGENS

One of the major sources for constant antigenic stimulation of the human immune system is the multiple bacteria that naturally colonize the GI tract. There are at least 400 different strains of bacteria in the GI tract, and they comprise >25% of the fecal material (Stephen and Cummings, 1980; Gerritsen et al., 2011). These bacteria present a wide range of antigens that can stimulate the human immune system. The multiple different polysaccharides and oligosaccharides on these bacteria serve as a source for many carbohydrate antigens that continuously stimulate the immune system to produce a wide variety of anti-carbohydrate antibodies, without the need for active vaccination by the carbohydrate antigens, i.e., natural anti-carbohydrate antibodies (Wiener, 1951; Springer, 1971). Anti-Gal is one of these natural antibodies and it is produced in high amounts throughout life (Galili et al., 1984; Wang et al., 1995). Anti-Gal was shown to bind to several GI bacteria, as well as to their lipopolysaccharide extracts, including *Klebsiella pneumoniae*, *Serratia marcescens*, and *Escherichia coli O86* (Galili et al., 1988b). In earlier studies, feeding killed *E. coli O86* bacteria to patients with diarrhea was found to result in significant increase in the titer of anti-blood group B antibodies (Springer and Horton, 1969). As detailed in Chapter 3, >85% of anti-blood group B antibodies in humans are in fact anti-Gal antibodies that can also bind to blood group B antigen (Galili et al., 1987b). Accordingly, feeding α1,3galactosyltransferase knockout mice (GT-KO mice) with *E. coli O86* was found to induce production of the anti-Gal antibody in these mice (Posekany et al., 2002). Furthermore, production of anti-Gal in monkeys could be inhibited by administration of antibiotics that eliminate the GI bacterial flora (Mañez et al., 2001).

Although the natural anti-carbohydrate antibodies are primarily produced against bacterial carbohydrate antigens, some of these antibodies are capable of binding to mammalian carbohydrate antigens, as well (Blixt et al., 2004; Bovin et al., 2012; Bovin, 2013; Stowell et al., 2014). Accordingly, the natural anti-Gal antibody is produced against bacterial carbohydrate antigens with terminal galactosyl units linked in an alpha anomeric linkage and is capable of binding to the mammalian α-gal epitope (Galili et al., 1985; Towbin et al., 1987). Anti-Gal binds to various bacteria and bacterial lipopolysaccharides (Galili et al., 1988b); however, the exact structure of bacterial carbohydrates inducing anti-Gal production has not been identified, as yet. Galα1-3Glc and Galα1-3Gal epitopes were reported on both gram-positive and gram-negative bacteria (Han et al., 2012; Lüderitz et al., 1965). Additional examples of such antibodies in humans are anti-blood group A and B antibodies (Springer and Horton, 1969), natural antibody to *N*-glycolylneuraminic acid (called anti-Neu5Gc), which is produced in humans, and not in other Old World primates, or in nonprimate mammals (Higashi et al., 1977; Merrick et al., 1978; Zhu and Hurst, 2002; Padler-Karavani et al., 2008), and natural anti-rhamnose antibody (Chen et al., 2011; Sheridan et al., 2014; Long et al., 2014). As detailed in Chapter 3, the reason for the ability of anti-bacterial carbohydrate antibodies to bind mammalian carbohydrate antigens is that these antibodies are polyclonal, and different clones are capable of binding to various "facets" of a given carbohydrate antigen. Some of these facets are likely to be shared between mammalian and bacterial carbohydrate antigens. As discussed below, mammals produce anti-carbohydrate antibodies against many carbohydrate epitopes, provided that these epitopes are not self-antigens. Thus, if for any reason, a mammal stops synthesizing a certain carbohydrate epitope, there is high probability that it

may start producing a natural antibody against that eliminated epitope, as part of the ongoing immune response against the many carbohydrate antigens on the bacteria of its natural GI flora.

Immune tolerance and anti-carbohydrate antibodies

The one factor that limits the diversity of anti-carbohydrate antibodies is the immune tolerance that prevents production of antibodies to self-carbohydrate antigens. Such production is prevented primarily by two mechanisms: (1) clonal deletion of immature B cell clones with B cell receptors, which can interact with self-antigens and (2) receptor editing in which the variable regions of immunoglobulin genes encoding antibodies to self-antigens are mutated so that the antibodies produced do not bind to self-antigens. The role of these mechanisms in prevention of anti-Gal production in animals synthesizing the α-gal epitopes as self-antigen was demonstrated in transgenic wild-type and GT-KO mice. Experimental studies in GT-KO mice indicated that the immune tolerance to the α-gal epitope is mediated by clonal deletion in which anti-Gal B cell clones, even at the stages of mature and memory B cells, are deleted following interaction of their B cell receptors with α-gal epitopes as self-antigen (Ogawa et al., 2003; Mohiuddin et al., 2003; Galili, 2004). Receptor editing mediating tolerance to α-gal epitopes was also observed in GT-KO mice in which an anti-Gal encoding gene was introduced (i.e., "knocked in") (Benatuil et al., 2008). These mice continuously produce anti-Gal without the need for their immunization. When such mice also acquired the active α1,3GT gene from wild-type mice, they ceased to produce anti-Gal because the variable regions of immunoglobulin genes encoding for anti-Gal B cell receptors were mutated at early stages of B cell development in the bone marrow. These mutations resulted in changes in the B cell receptor specificity, so it does not interact with α-gal epitopes (Benatuil et al., 2008). These immune tolerance mechanisms imply that once α-gal epitopes (and possibly other carbohydrate antigens) are eliminated because of inactivation of the gene encoding the corresponding glycosyltransferase, the immune tolerance mechanisms preventing production of antibodies against that self-antigen cease to function. Thus, the immune system is stimulated by bacteria of the GI flora to produce antibodies against the eliminated self-antigen. A present day example of a scenario in which a glycosyltransferase gene is inactivated in small human populations, and the resulting production of a natural antibody against the eliminated carbohydrate antigen is the blood group "Bombay" individuals, discussed at the end of this chapter. These rare individuals lack the blood group H (O) antigen and produce natural antibodies against this antigen.

A specific present day example for *de novo* production of the natural anti-Gal antibody following the elimination of α-gal epitopes was observed in recent years in α1,3GT knockout pigs (GT-KO pigs). As discussed in Chapter 1, these pigs were generated by disruption ("knockout") of the α1,3GT gene, with the aim of providing pig xenograft organs and tissues that lack α-gal epitopes (Lai et al., 2002; Phelps et al., 2003; Kolber-Simonds et al., 2004; Takahagi et al., 2005). Wild-type pigs present multiple α-gal epitopes as self-antigen on their cells, and thus, immune tolerance mechanisms prevent production of anti-Gal antibodies in them. However, once the α-gal epitope is eliminated by "knockout" of the α1,3GT gene in the GT-KO pig genome, these pigs naturally produce anti-Gal in high titers against GI bacteria, already at the age of 1.5–2 months (Dor et al., 2004; Fang et al., 2012; Galili, 2013). As discussed below,

a similar production of anti-Gal in primates, in which the α1,3GT gene was inactivated by mutations, might have prevented the extinction of Old World primates that were exposed to highly detrimental enveloped viruses or other pathogens expressing α-gal epitopes.

POSSIBLE EVOLUTIONARY APPEARANCE OF ANTI-GAL PRODUCING PRIMATES FOLLOWING VIRAL EPIDEMICS

The selective process that eliminated α-gal epitopes from ancestral Old World monkeys and apes (Old World primates) and led to the appearance of anti-Gal producing primates occurred after Old World primates and New World monkeys diverged from a common ancestor. Studies on the mutations inactivating the α1,3GT gene suggest that this selective process initiated 20–30 mya (see below). The lack of α-gal epitopes and production of the anti-Gal antibody are uniformly observed in monkeys and apes, which evolved in all regions of the Old World (i.e., the geographic area of Eurasia-Africa). The occurrence of this selective process throughout the vast regions of Eurasia-Africa suggests that it was mediated by a highly detrimental pathogen, such as enveloped virus (Galili, 2016). Influenza virus is one current example of a virus, which potentially can become highly virulent, causing lethal infections and can effectively spread throughout human populations. Intercontinental transportation can further enable its spread over geographic barriers. Because enveloped viruses lack their own glycosylation machinery, they share the carbohydrate antigens on their envelope glycoproteins with the host cell. Many of the glycosyltransferases within the host cells reside in the Golgi apparatus. They synthesize the carbohydrate chains on cellular and viral glycoproteins in a manner similar to assembly lines in a car plant, in that there is a sequential buildup of the nascent carbohydrate chain at various compartments of the Golgi apparatus. Therefore, enveloped viruses infecting primates that synthesize α-gal epitopes are likely to have these epitopes on their envelope glycoproteins.

Hypothesis on virus-mediated selection for elimination of α-gal epitopes in primates

The proposed scenario for elimination of α-gal epitopes in Old World primates and the resulting appearance of the natural anti-Gal antibody is based on the assumption that very rare mutation event(s) occurred accidentally and randomly in one or more of ancestral Old World primate species. Such a mutation could be single base frameshift deletion resulting in a premature stop codon, which completely inactivated α1,3GT catalytic activity. Accordingly, a three amino acid deletion at the C-terminus of New World monkey α1,3GT was found to result in complete loss of catalytic activity of the enzyme (Henion et al., 1994). It is likely that offspring carrying such a mutation for several generations after it occurred were heterozygotes. They produced intact α1,3GT by the unmutated allele and synthesized α-gal epitopes (see the description of these mutations below). However, the mating of such heterozygotes resulted in homozygous offspring primates that carried two alleles of the inactivated α1,3GT gene, and therefore they lacked α-gal epitopes. As the α-gal epitope in these homozygotes became a nonself antigen, they naturally produced the anti-Gal antibody in response to the constant antigenic stimulation by carbohydrate antigens with

structures similar to that of the α-gal epitope, presented on GI bacteria. These anti-Gal producing primates could evolve because the α-gal epitope turned out to be a nonessential carbohydrate epitope, similar to the observations with GT-KO pigs (Phelps et al., 2003; Kolber-Simonds et al., 2004). A scenario similar to the hypothetical one described above is presently observed in individuals of the rare blood group "Bombay" who lack the enzyme producing blood group O (H) and who naturally produce anti-H (blood group O) antibodies (Bhende et al., 1952; Watkins, 1980; Le Pendu et al., 1986; Balgir, 2005, 2007). The similarities between ancestral primate populations including small numbers of individuals lacking α-gal epitopes, prior to extinction of populations synthesizing α-gal epitopes, and present day human populations including small numbers of individuals lacking the ability to produce blood group O (i.e., blood group Bombay individuals) are further discussed at the end of this chapter.

As proposed in Fig. 2, early ancestral Old World primates synthesized α-gal epitopes similar to New World monkeys. These Old World primates could become extinct in epidemics of highly virulent enveloped viruses because they succumbed to the infections before these primates could mount a protective immune response against the infecting virus. The viruses mediating such epidemics carried α-gal epitopes on their envelope glycoproteins,

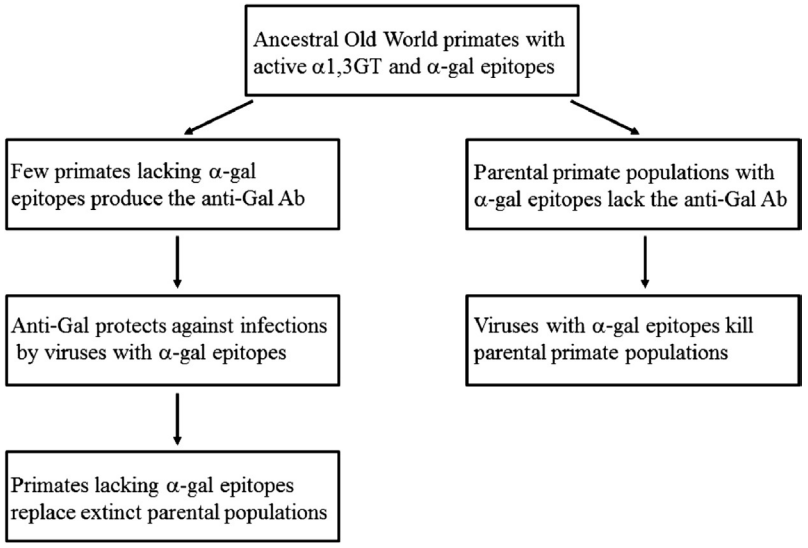

FIGURE 2 Proposed stages in the evolutionary selective process that resulted in elimination of ancestral Old World primates synthesizing α-gal epitopes and their replacement with offspring-lacking the α-gal epitope and producing the natural anti-Gal antibody. Few individuals in early Old World primate populations, who carried mutations inactivating the α1,3GT gene, produced the natural anti-Gal antibody. This antibody production is analogous to the present day rare blood type "Bombay" individuals lacking blood group O (H antigen) and producing anti-H antibodies. Epidemics by enveloped viruses presenting α-gal epitopes that were synthesized by α1,3GT of ancestral Old World primates caused the extinction of these primates, whereas offspring-lacking α-gal epitopes were protected by the natural anti-Gal antibody they produced. These offspring ultimately replaced the extinct primates that conserved active α1,3GT. Ab-antibody. *Reprinted from Galili, U., 2016. Natural anti-carbohydrate antibodies contributing to evolutionary survival of primates in viral epidemics? Glycobiology 26, 1140–1150, with permission.*

which were synthesized by α1,3GT in the infected host cells. However, the very few primates that were homozygous for the inactivated α1,3GT gene, lacked active α1,3GT enzyme, did not synthesize α-gal epitopes and produced the natural anti-Gal antibody. These few primates could have been protected by this antibody against viruses expressing α-gal epitopes. Anti-Gal protection could be mediated by several mechanisms, including: (1) neutralization and destruction of the virus by anti-Gal binding to the virus α-gal epitopes and activating the complement system, which induced complement-mediated lysis of the virus, (2) opsonization of the virus by anti-Gal could induce effective uptake and destruction of the virus by macrophages following Fc/Fcγ receptor interaction between the opsonizing anti-Gal and these cells, and (3) extensive uptake of anti-Gal opsonized viruses by macrophages and dendritic cells via Fc/Fcγ receptor interaction could result in rapid processing and presentation of immunogenic viral peptides by these antigen presenting cells (APC) that effectively transport the virus antigens to regional lymph nodes. This APC-mediated mechanism would have resulted in induction of rapid, potent humoral and cellular protective anti-virus immune responses. Thus, such an immune response could also protect against infecting viruses that "lost" their α-gal epitopes because of the initial infection of the host cells lacking α1,3GT. The ability of anti-Gal to markedly increase immunogenicity of vaccinating viruses by targeting them for effective uptake by APC is further detailed in Chapter 9 that describes the amplification of virus vaccine immunogenicity by α-gal epitopes linked to vaccinating viral glycoproteins (Abdel-Motal et al., 2006, 2007, 2010). Overall, the combined effects of the anti-Gal-mediated protective mechanisms could result in decrease in initial infecting virus burden, T cell-mediated destruction of cells infected by the virus, as well as destruction and neutralization of virus *de novo* produced in infected cells by elicited antibodies specific to virus protein antigens. The outcome of these protective mechanisms could be prevention of infective virus progression before it reaches lethal stages.

Anti-Gal IgG crosses the placenta into the fetal blood in humans (Galili et al., 1984). Anti-Gal is also present in colostrum and milk, as well as in other body secretions, primarily as the IgA isotype (class) (Hamadeh et al., 1995). Thus, it is possible that anti-Gal-mediated protection against an infectious virus that presents α-gal epitopes also occurred in newborns. In the absence of competition from parental primate populations synthesizing α-gal epitopes, the small populations of offspring-lacking α-gal epitopes and producing the natural anti-Gal antibody replaced the extinct parental Old World primate populations that conserved active α1,3GT. It should be stressed that the proposed scenario could occur with any type of enveloped virus that presented α-gal epitopes, which was endemic to the Eurasia-Africa landmass because any enveloped virus propagated in cells containing active α1,3GT is likely to present α-gal epitopes. The process of selective evolutionary elimination of α-gal epitopes, which occurred in ancestral Old World primates, may not be feasible in all mammalian species synthesizing α-gal epitopes. One example is GT-KO mice. These mice develop cataract at the age of 6–9 weeks in the absence of α-gal epitopes (Thall, 1999; Sørensen et al., 2008). Although GT-KO mice developing such cataract can survive in the protected environment of animal facilities, their survival would have been questionable in natural environments. In contrast, GT-KO pigs were not reported to develop the cataract observed in mice in the absence of α-gal epitopes.

Observations that support the hypothesis on evolution associated with viral epidemics

As indicated above, it is impossible to identify a pathogen(s) that exerted the selective pressure for evolution of primates lacking α-gal epitopes and producing the anti-Gal antibody in earlier geological periods. However, there are several observations supporting the hypothesis proposed in Fig. 2.

Anti-Gal interaction with viruses carrying α-gal epitopes

Glycoproteins are an integral part of virus envelopes. The carbohydrate chains on such glycoproteins contribute to the formation of a hydration layer that protects the virus. Carbohydrate chains of the complex type of glycoproteins (Fig. 2A in Chapter 1) are synthesized on aspargines (-*N*-) that are part of the amino acid sequence -*N*-X-S/T- in proteins. Because these carbohydrate chains are synthesized by the host cell glycosylation machinery, viruses propagated in cells containing α1,3GT usually present multiple α-gal epitopes. Thus, propagation of Eastern Equine Encephalitis virus in mouse cells resulted in production of virions carrying α-gal epitopes, whereas propagation of this virus in African Green monkey Vero cells (lacking active α1,3GT) resulted in production of virions with envelope glycoproteins lacking α-gal epitopes (Repik et al., 1994). Accordingly, influenza virus propagated in embryonated chicken eggs lacks α-gal epitopes because birds, as other nonmammalian vertebrates, lack α1,3GT. In contrast, propagation of influenza virus in bovine MDBK cells or canine MDCK cells resulted in production of virions with the envelope glycoprotein hemagglutinin carrying several α-gal epitopes per molecule (Galili et al., 1996).

α-Gal epitopes were also demonstrated on other viruses propagated in nonprimate mammalian cells, including: Friend murine leukemia virus (Geyer et al., 1984), murine Molony leukemia virus (Rother et al., 1995), porcine endogenous retrovirus (PERV) (Takeuchi et al., 1996), lymphocytic choriomeningitis virus, Newcastle disease virus, Sindbis virus, vesicular stomatitis virus (Welsh et al., 1998), and measles virus (Preece et al., 2002; Dürrbach et al., 2007). Several of these studies further showed that incubation of the viruses expressing α-gal epitopes in human serum or with purified anti-Gal antibody further resulted in anti-Gal-mediated neutralization and complement-mediated lysis of the viruses, whereas no such effects were observed in viruses lacking α-gal epitopes. As suggested in Fig. 2, it may be possible that a similar protective effect was mediated by anti-Gal in the few individuals among Old World primates that had mutations inactivating the α1,3GT gene. In contrast, populations conserving α1,3GT activity produced virions presenting α-gal epitopes and were killed by such viruses in the absence of anti-Gal. The observed anti-Gal-mediated destruction and neutralization of viruses carrying α-gal epitopes further suggested that this antibody may contribute to prevention of cross-species viral transmission from nonprimate mammals to humans (Repik et al., 1994; Rother et al., 1995; Takeuchi et al., 1996; Welsh et al., 1998; Preece et al., 2002).

Increased protective immune response by anti-Gal targeting of viruses to antigen presenting cells

As detailed in Chapter 9, anti-Gal-mediated targeting to APC of inactivated influenza virus engineered to present α-gal epitopes was found to increase anti-virus antibody response in GT-KO mice by ~100-fold, in comparison with mice immunized with inactivated influenza

virus lacking α-gal epitopes (Abdel-Motal et al., 2007). Intranasal challenge of the immunized mice with a lethal dose of live influenza virus lacking α-gal epitopes resulted in death of 90% of mice immunized with virus lacking α-gal epitopes, whereas only 10% of mice immunized with virus presenting α-gal epitopes died after such challenge (Abdel-Motal et al., 2007). Similarly, anti-Gal-producing GT-KO mice immunized with gp120 of HIV carrying α-gal epitopes resulted in ~100-fold higher anti-gp120 antibody response, ~30-fold higher T cell response, and ~40-fold increase in *in vitro* HIV neutralization activity in comparison to the immune responses measured in mice immunized with gp120 lacking α-gal epitopes (Abdel-Motal et al., 2006). A similar increase in anti-virus CD8+ cytotoxic T cell response was reported in anti-Gal-producing GT-KO mice that were immunized with a mouse cell line expressing murine leukemia virus proteins and α-gal epitopes, in comparison with CD8+ T cell response in wild-type mice (i.e., mice lacking the anti-Gal antibody) and undergoing similar immunization (Benatuil et al., 2005). All these studies support the assumption that ancestral Old World primates lacking α-gal epitopes and producing the anti-Gal antibody could enhance the immune response against proteins of infecting viruses presenting α-gal epitopes, by anti-Gal-mediated targeting of the virus to APC. The enhanced immune response might have been potent enough to prevent progression of the infection to lethal stages even when the virions lacked α-gal epitopes because of growth in cells lacking active α1,3GT in anti-Gal producing hosts.

Enveloped viruses appearing after New World monkeys/Old World primates split

The hypothesis on the role of enveloped viruses in mediating the selective pressure for evolution of primates lacking α1,3GT and producing anti-Gal, includes the assumption that such viruses appeared in the Old World only after the split from New World monkeys, i.e., New World monkeys were geographically isolated in the South American continent and thus were not affected by these viruses. This assumption is supported by observations of an enveloped virus, Epstein Barr virus (EBV), which is of the Herpes virus family, and it is thought to have appeared among Old World primates after the geographic separation from New World monkeys. Therefore, this virus has influenced immune system evolution of only Old World primates. When EBV infects Old World primates, it immortalizes a proportion of their B cells. However, the immune system in Old World primates evolved to mount an extensive T cell response against EBV antigens, which in humans results in the transient infectious mononucleosis disease (Klein and Masucci, 1982; Callan, 2003). The proliferating T cells kill the majority of B cells infected by the virus and immortalized. Moreover, EBV-immortalized B cells residing in immunologic sanctuaries are destroyed upon detection by T cells if they leave such sanctuaries. As many as 90% of humans are infected by EBV. However, because of the effective T cell response against EBV infected B cells; these B cells are prevented from spreading throughout the body and from progressing into becoming lymphoma cells. In contrast, no significantly effective anti-EBV protective T cell response is observed in New World monkeys infected by EBV because the immune system in these primates was not evolutionarily exposed to infections by this virus. Therefore, many of the EBV-immortalized B cells in New World monkeys are not destroyed and progress into lethal polyconal B cell lymphomas (Epstein et al., 1973; Shope et al., 1973; Wang, 2013). Elimination of α-gal epitopes and production of the natural anti-Gal antibody may represent an analogous selective pressure mediated by a virus endemic to the Old World land mass, whereas New World monkeys

evolving in South America or lemurs evolving in Madagascar have not been subjected to evolutionary selective processes by such hypothetical viruses because of geographic isolation from the Old World.

Evolutionary almost complete extinction of apes according to their fossil record

The occurrence of mutations that result in elimination of major cell surface antigens, such as the α-gal epitope and the appearance of a natural antibody against it, are very rare events in evolution. There is only one other similar event known in the evolution of Old World primates, the elimination of the sialic acid *N*-glycolylneuraminic acid (Neu5Gc) in hominins (ancestors of humans) and production of natural anti-Neu5Gc antibodies that are found only in humans (Zhu and Hurst, 2002; Padler-Karavani et al., 2008). The rest of Old World primates and nonprimate mammals synthesize Neu5Gc and lack anti-Neu5Gc antibodies (Varki, 2010). These selective processes were likely to be associated with extinction of the parental primate populations conserving the carbohydrate antigen and thus, lacking the natural antibody against it. Although it is practically impossible to associate between the fossil record from previous geological periods and biochemical/immunological changes in primate populations, there is an interesting parallelism between the suggested hypothesis on extinction of apes that conserved α-gal epitopes and the fossil record of apes. Apes were a very successful group of primates in the early Miocene (~20–23 mya) as implied from the multiple fossils of many ape species (*hominoidea*) dating to that period, which were found in Eurasia-Africa. However, the number and diversity of ape fossils from the middle Miocene (~11–16 mya) greatly declines. No fossils of apes from the late Miocene (~5–10 mya) have been found, suggesting an almost complete extinction of apes at that period (Andrews, 1992; Andrews et al., 1996; Merceron et al., 2010; Alba, 2012). These changes in ape populations have been associated with dietary adaptations because of climatic changes (Andrews and Martin, 1991; Agustí et al., 2003; Ungar and Kay, 1995). An alternative cause for this almost complete extinction of ancestral apes could be associated with the selective pressure for the evolution of apes lacking α-gal epitopes and producing the anti-Gal antibody (Galili and Andrews, 1995), possibly mediated by epidemics of viruses carrying α-gal epitopes, as suggested above. The slow decline in ape populations during the middle Miocene, toward their almost complete extinction in the late Miocene, may further suggest that the extinction of primates by viral epidemics and expansion of subpopulations lacking α-gal epitopes throughout Eurasia-Africa could have been slow processes taking millions of years. The slow pace of these changes may have been the result of the great geographical distances between various ape populations. The fossil record of Old World monkeys dating to those periods is sparse, and thus, it is difficult to determine the population changes in this group of primates during the Miocene (Miller et al., 2009).

ALTERNATIVE CAUSES FOR EVOLUTIONARY INACTIVATION OF α1,3GT IN ANCESTRAL OLD WORLD PRIMATES

The efficacy of anti-Gal in protecting against infections with viruses presenting α-gal epitopes may vary for different enveloped viruses. One example for insufficient protective activity is that of influenza virus. As indicated above, when this virus is grown is cells that have active α1,3GT (e.g., bovine MDBK cells or canine MDCK cells), the virus carries α-gal epitopes

on its hemagglutinin envelope protein (Galili et al., 1996). Thus, it is likely to carry α-gal epitopes also when produced in porcine cells. Indeed PERV grown in porcine cells can be destroyed by anti-Gal in human serum (Takeuchi et al., 1996). Nevertheless, humans can be infected by influenza virus produced in pigs. Once few virions succeed in penetrating into human cells of the respiratory tract, they proliferate and carry carbohydrate chains produced by the human glycosylation machinery, i.e., chains lacking α-gal epitopes. Thus, additional scenarios for the evolutionary processes that could result in extinction of ancestral Old World primates presenting α-gal epitopes should be considered, as well. Three of these scenarios are as follows:

1. *Detrimental bacteria expressing α-gal-like epitopes*—Several bacterial strains bind the anti-Gal antibody (Galili et al., 1988b), provide antigens that elicit production of anti-Gal in humans (Almeida et al., 1991) and in GT-KO mice (Posekany et al., 2002), and display carbohydrate antigens with terminal α-galactosyls in both gram-negative and gram-positive bacteria (Lüderitz et al., 1965; Han et al., 2012). It could be hypothesized that bacterial strains that were lethal to Old World primates, which expressed antigens that elicit anti-Gal production, could generate a selective pressure for survival of primates that produced this antibody as a protective antibody, i.e., selection for individuals with inactivated α1,3GT gene.
2. *Bacterial toxins or viruses binding the α-gal epitope*—An alternative hypothesis involving bacteria could be that the lethal effects of the infecting bacteria were mediated by binding of their toxins to α-gal epitopes on host cells. A current example is enterotoxin A of *Clostridium difficile* that causes severe diarrhea. This toxin can bind to various carbohydrate receptors; however, its primary receptor on nonprimate mammalian cells is the α-gal epitope (Pothoulakis et al., 1996; Teneberg et al., 1996). It may be possible that epidemics among Old World primates by bacteria producing lethal toxin(s) that used α-gal epitopes as receptor on target cells, exerted a selective pressure for survival of individuals that lacked the α-gal epitopes and thus, were not affected by the toxin. As discussed above, once the α-gal epitope was eliminated, the immune tolerance to this antigen was lost, resulting in production of the natural anti-Gal antibody. A similar selective process may be envisaged if a detrimental virus "used" the α-gal epitope as a "docking receptor." Influenza virus uses sialic acid on cells as a docking receptor that enables it to attach to cell membranes and penetrate into cells. If there was a virus that used the α-gal epitope as such a receptor, it could drive the selection of primates to survival only of those lacking α-gal epitopes along a pathway similar to that described in Fig. 2, without the involvement of antibodies in the selective process. However, anti-Gal production would have been a by-product resulting from the loss of the α-gal epitope and of the immune tolerance to it. A current example for such a virus is bovine norovirus that was reported to use α-gal epitopes as a docking receptor for infecting bovine cells (Zakhour et al., 2009). In addition, Sindbis virus was found to preferentially infect cells that present α-gal epitopes and wild-type suckling mice synthesizing this epitope, in comparison with cells or suckling mice lacking α-gal epitopes (Rodriguez and Welsh, 2013).
3. *Detrimental protozoa that express α-gal or α-gal-like epitopes*—As discussed in detail in Chapter 4, several protozoa, which are parasitic in humans, were found to present cell surface carbohydrate epitopes with structures similar to the α-gal epitope. These include *Trypanosoma* (Ramasamy and Field, 2012; Milani and Travassos, 1988; Almeida et al., 1994), *Leishmania* (Avila et al., 1989; McConville et al., 1990; Ilg et al., 1992), and *Plasmodia*

(Ramasamy and Reese, 1986; Yilmaz et al., 2014). As argued above for bacteria, such protozoa pathogens could mediate the selective pressure for survival of individuals in which α-gal epitopes were eliminated and the natural anti-Gal antibody produced (Ramasamy and Rajakaruna, 1997; Yilmaz et al., 2014). These antibodies could serve as protective antibodies against infections by protozoa presenting anti-Gal-binding epitopes. Indeed, anti-Gal binding to *Trypanosoma cruzi* was shown to induce complement-mediated cytolysis of the parasite (Milani and Travassos, 1988; Almeida et al., 1991, 1994) as well as direct, complement-independent lysis (Gazzinelli et al., 1991).

Although bacteria and protozoa epidemics cannot be excluded as evolutionary causes for selection of Old World primates with inactivated α1,3GT gene, the likelihood of these scenarios may be lower than the scenario of enveloped viruses mediating such a selective pressure. The complete elimination of Old World monkeys and apes producing α-gal epitopes in all climatic regions of Eurasia-Africa suggests that the pathogen(s) had to have very high infectivity and may have not depended on secondary transmitting vectors (e.g., insects active only in certain climates). Such are characteristics of viruses that spread directly from one infected individual primate to the other, regardless the large variety of climatic environments.

MOLECULAR BASIS FOR THE EVOLUTIONARY INACTIVATION OF THE α1,3GT GENE

The elimination of the α-gal epitope in ancestral Old World primates was the result of mutations that inactivated the α1,3GT gene (*GGTA1*) in few individuals, and subsequently in small populations of ancestral primates. α1,3GT activity was not essential in ancestral primates who were homozygous for the inactivated α1,3GT gene (Galili et al., 1987a, 1988a). These observations raised the question of the mechanism that inactivated α1,3GT gene in Old World primates. This question could be addressed following the cloning of the α1,3GT gene in mouse and bovine cells (Larsen et al., 1989; Joziasse et al., 1989). The gene was found to be composed of ~1110 base pairs divided into nine exons, of which exon IX (687 bp) is the largest.

Comparison of DNA and derived protein sequences of exon IX in mouse and bovine α1,3GT and a cloned homologous human genomic sequences, indicated that in the human DNA sequence, there are two frameshift mutations caused by single base deletions, corresponding to base 822 and base 904 of the mouse α1,3GT cDNA (Fig. 3) (Larsen et al., 1989, 1990; Joziasse et al., 1989; Lantéri et al., 2002). These mutations create premature stop codons, truncating the α1,3GT enzyme by 110 and 15 amino acids at the C-terminus, respectively. Controlled truncation of a New World monkey α1,3GT cDNA indicated that elimination of as few as the last three amino acids at the C-terminus of the enzyme was sufficient to cause complete loss of catalytic activity of α1,3GT (Henion et al., 1994). This implies that α1,3GT gene in humans is a pseudogene incapable of producing an active enzyme. Sequencing of the homologous DNA region in apes revealed that orangutan and gorilla have an α1,3GT pseudogene containing only one of the two deletions, at base 904, whereas chimpanzee has both deletions, similar to humans (Fig. 3) (Galili and Swanson, 1991). The absence of any of these two deletions in Old World monkey α1,3GT pseudogenes (Rhesus, African green, and Patas monkeys in Fig. 3) suggested that the deletions appeared in apes after they and Old World monkeys diverged from a common ancestor, i.e., less than 28 mya. However, a third

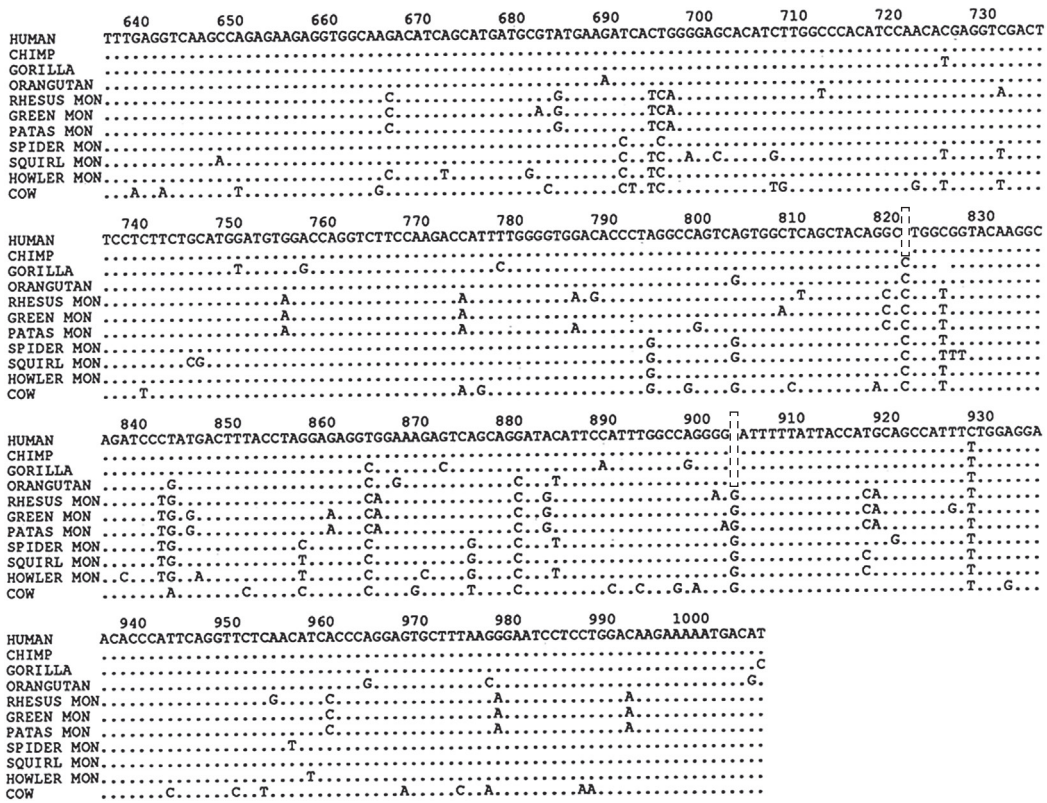

FIGURE 3 Aligned DNA sequences of a 370-bp region in exon IX of the αl,3GT pseudogene from humans (Larsen et al., 1990), apes including: chimpanzee, gorilla and orangutan, and Old World monkeys including: Rhesus monkey, African green monkey, and Patas monkey. These sequences are aligned with the active αl,3GT gene in New World monkeys including: Spider monkey, Squirrel monkey, and Howler monkey, and with domestic cow (described by Joziasse et al., 1989). The base numbers in this figure are according to the open reading frame of the mouse αl,3GT cDNA described by Larsen et al. (1989). The numbered base is under the second digit. Dots represent sequences identical to those of the human αl,3GT pseudogene. Note the two deletions C822 and G904 in humans and chimpanzees and G904 in humans and apes but not in other primates. *Reprinted from Galili, U., Swanson, K., 1991. Gene sequences suggest inactivation of α1-3 galactosyltransferase in catarrhines after the divergence of apes from monkeys. Proc. Natl. Acad. Sci. U.S.A. 88, 7401–7404, with permission.*

deletion was found in exon VII in rhesus monkey, in orangutan, and in humans (Koike et al., 2002). This mutation in an Old World monkey, ape, and humans suggested that the inactivation of the gene occurred before divergence of Old World monkeys and apes from a common ancestor (Koike et al., 2002). Based on these studies, it is not clear at present whether the selective process for extinction of Old World primates synthesizing the α-gal epitope and the emergence of primates lacking this epitope and producing anti-Gal, initiated before or after the split between apes and monkeys of the Old World, and thus, it is considered to initiate 20–30 mya (Fig. 1).

BLOOD GROUP BOMBAY AS A PRESENT DAY EXAMPLE FOR A RARE GLYCOSYLTRANSFERASE INACTIVATION IN HUMANS

The basic assumption in regard to the evolutionary elimination of ancestral Old World primates producing α-gal epitopes is that prior to this elimination there have been few primates that were homozygous for mutations that inactivated the α1,3GT gene (*GGTA1*) and thus produced the natural anti-Gal antibody. This assumption is supported by a similar present day example of a very rare mutation in humans, which inactivates the α1,2 fucosyltransferase gene (α1,2FT also called *FUT1*). Individuals homozygous for this mutation belong to blood group "Bombay" and are characterized by inability to synthesize the H antigen (Fucα1-2Gal-R that is blood group O) (Bhende et al., 1952; Watkins, 1980; Le Pendu et al., 1986; Balgir, 2005, 2007). These individuals are designated as Oh or h/h, in contrast to most humans who are H/H, i.e., they produce the blood group O carbohydrate antigen. In individuals who are blood group A or B, *N*-acetylgalactosamine (GalNAc) or galactose (Gal) is added α1-3 to the penultimate Gal of the H antigen, respectively. The structure of blood type "Bombay" antigen Oh is included in Fig. 1 of Chapter 3 that illustrates the α-gal epitopes and blood type ABO antigens, as well. Cloning and sequencing of the α1,2FT gene (*FUT1*) in blood group Bombay (Oh) individuals demonstrated the presence of inactivating point mutations in the coding regions of both alleles of this gene (Kelly et al., 1994; Fernandez-Mateos et al., 1998).

Blood group Bombay individuals are very rare. They are found in European populations as 1:1,000,000, whereas in India they are 1:10,000. In the absence of blood group O, blood group Bombay individuals naturally produce anti-blood group H (O) antibodies (i.e., anti-Fucα1-2Gal-R antibodies). These natural antibodies are completely absent in all other human populations. Blood group Bombay individuals also produce natural anti-A and anti-B antibodies because in the absence of blood group H, they cannot synthesize blood groups A or B antigens. Thus, blood group Bombay individuals resemble the hypothesized ancestral Old World primates who lived prior to the extinction of α-gal epitopes synthesizing primates, in the following characteristics: (1) Individuals who are homozygous for the accidently acquired mutation(s) that inactivated the α1,2FT gene, and those primates with inactivated α1,3GT gene have been very rare within their corresponding populations. (2) The homozygous individuals for the inactivated glycosyltransferase genes lack the H antigen or the α-gal epitope and produce a natural antibody against the lost carbohydrate antigen. As indicated above, anti-Gal can destroy or neutralize enveloped viruses presenting α-gal epitopes, following propagation in mammalian cells containing active α1,3GT. Similarly, enveloped viruses including severe acute respiratory syndrome coronavirus (Guillon et al., 2008), measles virus (Preece et al., 2002), and HIV (Neil et al., 2005) were found to present blood group A or B carbohydrate antigens when propagated in cells of containing α1,2FT and the corresponding A or B transferases. These viruses were further found to undergo complement-mediated inactivation in sera containing anti-A or anti-B antibodies, respectively. Thus, it would be of interest to determine whether anti-blood group H (O) antibody in the serum of blood group Bombay individuals can inactivate enveloped viruses propagated in blood group H human cells. Such an anti-viral activity of anti-blood group H (O) antibody raises a hypothetical possibility that blood group Bombay individuals may be immuno-protected better than other humans by the natural anti-blood group H (O), anti-A and anti-B antibodies against virulent enveloped viruses originating in any individual who is not blood group Bombay. This protection may be in a manner analogous to the effects of anti-Gal on enveloped viruses presenting α-gal epitopes, described in Fig. 2.

CONCLUSIONS

The natural anti-Gal antibody is one of the multiple natural anti-carbohydrate antibodies produced in humans against a wide range of carbohydrate antigens on GI bacteria. The antibody is unique to humans, apes, and Old World monkeys, and it binds specifically to a mammalian carbohydrate antigen called the α-gal epitope that is synthesized in nonprimate mammals, lemurs (prosimians) and New World monkeys by the glycosylation enzyme α1,3GT. The α1,3GT gene (*GGTA1*) appeared in mammals >100 million years ago, prior to the split between marsupial and placental mammals. This gene has been conserved in its active form, in all mammals, except for Old World monkeys, apes, and humans. Inactivation of the α1,3GT gene in ancestral Old World primates occurred 20–30 million years ago and could have been associated with epidemics of enveloped viruses in the Eurasia-Africa continent. It is suggested that prior to such epidemics, few ancestral Old World primates acquired deletion point mutations that inactivated the α1,3GT gene and eliminated α-gal epitopes. This resulted in loss of immune tolerance to the α-gal epitope and thus, in production of the anti-Gal antibody against antigens on bacteria colonizing the GI tract. This accidental inactivation of the α1,3GT gene in very small populations is analogous to the highly rare blood type "Bombay" individuals who do not synthesize blood group H (O antigen) because of inactivation of the α1,2-fucosyltransferase gene. The loss of immune tolerance to blood group H antigen has resulted in production of natural anti-blood group H antibodies in the blood group Bombay individuals. It is suggested that anti-Gal protected against infections by enveloped viruses presenting α-gal epitopes, which were lethal to the parental primate populations that conserved active α1,3GT and thus, synthesized α-gal epitopes. Alternative causes for the elimination of Old World primates synthesizing α-gal epitopes could be bacteria or protozoa parasites presenting α-gal or α-gal-like epitopes, and bacterial toxins, or detrimental viruses that used α-gal epitopes in these primates as "docking receptors." Ultimately, any of these proposed selective processes could result in extinction of Old World primates synthesizing α-gal epitopes on their cells. These ancestral primates were replaced by offspring populations lacking α-gal epitopes and producing the anti-Gal antibody, which continues to be produced by Old World monkeys, apes, and humans. New World monkeys and lemurs were protected from pathogens of the Old World by oceanic barriers, thus they continue to synthesize α-gal epitopes and lack the ability to produce the anti-Gal antibody. This scenario of few individuals in a large population having a mutation(s) that inactivates a glycosyltransferase gene thus, resulting in production of evolutionary advantageous natural antibodies against the eliminated carbohydrate antigen, may reflect one of the mechanisms inducing changes in the carbohydrate profile of various mammalian populations.

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